REMARKS

Claim 9 has been amended to depend from claim 27 instead of claim 1.

Figure 5 has been amended as suggested by the Examiner to change "Dia" to "Day." A replacement figure with this change is attached.

Applicants submit that the above amendments do not add any new matter, and their entry is requested.

Applicants affirm the election of Group I for examination and further confirm the election of species made during the telephone conversation with the Examiner on 3 April 2006.

The Examiner objected to Figure 5. It is believed that the amendment to Figure 5 obviates this objection, and its withdrawal is requested.

The Examiner rejected claim 9 under 35 U.S.C. § 112, second paragraph for being indefinite. It is believed that the amendment to claim 9 obviates this rejection, and its withdrawal is requested.

The Examiner rejected claims 1-10, 27 and 28 under 35 U.S.C. § 103 (a) as being obvious over Rodriguez et al. (US 5,788,985) in view of Hammonds et al. (US 4,857,637) and Udayachander et al. (*Human Antibodies* **8**:60-64, 1997). In essence, the Examiner cites Rodriguez et al. for its disclosure of the OMPC of *Neisseria meningitides* into which N-glycoyl GM3 has been incorporated (i.e., VSSPs) and its use for treating breast cancer. The Examiner cites Hammonds et al. for its disclosure of using EGFR (i.e., HER-1) as an antigen to immunize animals and for its disclsoure that EGFR is overexpressed in malignant cells and thus is a desirable target for therapy. Hammonds et al. also discloses the use of an adjuvant for immunization with growth factor receptors. Finally, the Examiner cites Udayachander et al. for its disclosure that many malignancies, such as breast cancer overexpress EGFR and that EGFR is a target for therapy. In view of these teachings in the art, the Examiner concludes that it would be *prima facie* obvious to combine the two treatments of Rodriguez et al. and Hammonds et al. into a single treatment for breast cancer because a skilled artisan would be motivated to use both compositions in combination in a method for treating a malignant tumor that overexpresses the

two antigens, such as breast cancer. However, as demonstrated below, it is submitted that the Examiner is in error in this rejection.

Specifically, Applicants submit that although Rodriguez et al. discloses VSSPs, there is no disclosure in Rodriguez et al. that the VSSPs can be utilized to potentiate the immunogenicity of low immunogenic antigens, such as growth factor receptors. In addition, there is no disclosure in any of the secondary references that the VSSPs of Rodriguez et al. could potentiate the immunogenicity of low immunogenic antigens. Because there is no suggestion in Rodriguez et al. that the immunogenicity of low immunogenic antigens, such as growth factor receptors, could be potentiated, there is no motivation to combine the references as proposed by the Examiner. There is no suggestion in either of the secondary references that the immunogenicity of the disclosed antigens should be potentiated. In the absence of any teaching in the primary or secondary references concerning potentiation of the immunogenicity of growth factor receptors, there is no motivation or suggestion in the cited art to combine these references in the manner proposed by the Examiner. Thus, Applicants submit that the cited prior art does not render the subject matter of claims 1-10, 27 and 28 obvious.

Furthermore, Rodriguez et al. discloses vaccine compositions for stimulating or increasing the immune antibody response to any ganglioside. It especially should be noted that Rodriguez et al. primarily discloses the obtainment of immunogens based on non-covalent coupling of the gangliosides to the outer membrane protein complex of Neisseria meningitidis, or the obtainment of immunogens based on covalent binding of the oligosaccharide component of the NGcGM3 ganglioside to a murine monoclonal antibody.

Moreover, Rodriguez et al. also teaches how IgG antibody specific responses against N-acetyl GM3, the most abundant ganglioside in normal tissues, can be consistently induced by immunization with the vaccine composition described in combination with an adjuvant. In another aspect, Rodriguez et al. also relates to the use of hybridoma biomass as a source of gangliosides.

On the other hand and surprisingly, Applicants have found that the claimed pharmaceutical composition of the present invention confers immunogenicity to peptides,

polypeptides, proteins and their corresponding DNA sequences (which are molecules completely different from the immunological point of view), and target cells of vaccine interest just by mixing them with the VSSPs described by Rodriguez et al. Furthermore, this technological solution allows the uses of the whole structure of the growth factor receptors, thereby solving the immune dominance genetic restriction. This feature of the VSSPs is not disclosed in Rodriguez et al. or in the secondary references.

As a difference from the cited prior art, the claimed pharmaceutical compositions of the present invention shows surprising immunological properties such as a dramatic ability to cause dendritic cells maturation and restoring immune-suppressed patients. Moreover, the claimed compositions of the present invention have the capacity of stimulating both humoral and cellular responses against a particular low immunogenic antigen.

Taking into account the very well known fact that the immune response generated by proteins is quite different from that generated by carbohydrates, this important feature of claimed compositions described of the present invention has been neither suggested nor anticipated by Rodriguez et al. or any other prior art.

Moreover, two main features distinguish the present invention from the cited prior art and from the state of art:

- An innovative aspect of the present invention is the rather small size of the particles in VSSP. It is very well known in the art the use of the outer membrane protein complex of Neisseria for obtaining immunogens. But what Applicants found was that using these nanoparticles the adjuvant capacity of the protein complex was substantially increased, with the ganglioside playing a crucial role in it.
- The other innovative aspect is relates to the biological functions of the ganglioside. Applicants note that gangliosides other than GM3 can be used in the present invention. The GM3 ganglioside is one of the most powerful immune suppressants. However, Applicants have found that the side effect targeting of GM3 in VSSP renders pharmaceutical compositions with the capacity of stimulating both humoral and cell responses, particularly in individuals with a deeply depressed immune

system, like patients with cancer or AIDS. In these individuals, the use of VSSP is reasonably a superior adjuvant solution than using just the natural outer membrane protein complex alone. In addition and particularly relevant for vaccine compositions including VSSP and EGF-R related polypeptides is the fact that GM3 co-modulates the signal transduction function of the receptor, once triggered by growth factors. Thus, the VSSP/EGF-R vaccine of the present invention is the only possible technical solution which resembles the real scenario of natural biological interactions of these molecules in the cells.

The Examiner stated that Hammonds et al. teaches a pharmaceutical composition further comprising HER-1 as an antigen to immunize animals against the EGF-R. In addition, The Examiner stated that Udayachander et al. teach that many malignancies over express EGF-R and suggest that it could be a target for cancer therapy. The Examiner is correct in her assertion that the EGF-R is a desirable target for cancer therapy. However, Applicants submit that in the field of cancer immunotherapy, the mere identification of a desirable target does not suggest any real probability in arriving at a result that will be useful.

Hammonds et al. teaches that immunizing an animal against a cell surface receptor or fragments thereof, coupled to a carrier protein, preferably KHL and using an adjuvant, can lead to the recovery of specific antibodies from the serum of the immunized animal or monoclonal antibodies from cell cultures. In Hammonds et al., the efficiency of the immune response is not crucial. In other words, even when the immune response against the specific antigen was low, lymphocytes producing antibodies against the antigen could be isolated. To the contrary, the present invention relates to a composition for increasing the immunogenicity of poorly immunogenic antigens.

Moreover, Hammonds et al.'s disclosure is primarily directed to growth hormone. Only Example 3 refers to the preparation of immunogens for raising EGF-R agonist antibodies. However, Hammonds et al. does not describe any immune response obtained when immunizing with the fragments.

The relevance of EGF/EGF-R system for tumour growth is very well known in the art, and that this over expression has been correlated with disease progression and poor prognosis. Although one of ordinary skill in the art may be motivated to use the two components in combination in a method of treating a malignant tumour that overexpresses the two antigens, there is no expectation of success in the art. For example, several approaches for passive therapy in which EGF-R has been targeted with monoclonal antibodies as well as for the treatment with tyrosine kinase inhibitor drugs, such as Iressa and Tarceva, have failed. Contrary to these failures in the prior art, the use of the claimed pharmaceutical compositions of the present invention for EGF-R based active immunotherapy is successful. The success of the present invention has recently been published in a peer-reviewed journal. See the attached abstract of the Ramirez, B.S. et al., "Active antimetastatic immunotherapy in Lewis lung carcinoma with self EGFR extracellular domain protein in VSSP adjuvant," Int J Cancer, Epub ahead of print 13 July 2006 (DOI: 10.1002/ijc22085). A copy of the accepted manuscript and figures is also attached for the convenience of the Examiner. This active immunotherapy approach and its success is not disclosed or suggested in any of the cited prior art alone or in combination. Thus, Applicants submit that the claimed invention is not obvious from the cited prior art.

In view of the above remarks, it is submitted that the present invention is not obvious over Rodriguez in view of Hammond et al. and Udayachander et al. Withdrawal of this rejection is requested.

The Examiner rejected claim 11 under 35 U.S.C. § 103 (a) as being obvious over Rodriguez et al. in view of Hammonds et al. and Udayachander et al. and in further view of Carr et al. (*Melanoma Research* **11**:219-227, 2001). Carr et al., which was published in June 2001, is cited for its disclosure of Montanide ISA51. However, as demonstrated above, it is submitted that the Examiner is in error in this rejection.

Specifically, the combination of Rodriguez et al., Hammonds et al. and Udayachander et al. does not render obvious the subject matter of claims 1-10, 27 and 28 as detailed above. Because the tertiary reference, Carr et al., does not supply any of the deficiencies of the cited primary and secondary references, it cannot render the subject matter of claim 11 obvious.

Furthermore, Carr et al. described the effect of the ganglioside vaccine on the B16 mouse melanoma experimental model. The vaccine composition used by Carr et al. is the same vaccine composition described in Rodriguez et al., therefore the same comments made above with respect to Rodriguez et al. also apply to Carr et al. Thus, Applicants submit that the subject matter of claim 11 is not obvious from Rodriguez et al., Hammonds et al. and Udayachander et al. further in view of Carr et al.

In view of the above remarks, it is submitted that the present invention is not obvious over Rodriguez in view of Hammond et al. and Udayachander et al. and further in view of Carr et al. Withdrawal of this rejection is requested.

In view of the above amendments and remarks, it is believed that the present claims satisfy the provisions of the patent statutes and are patentable over the cited prior art. Reconsideration of the application and early notice of allowance are requested. The Examiner is invited to telephone the undersigned to expedite the prosecution of the application.

Respectfully submitted,

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By _____

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Attachments: Revised Figure 5
Ramirez et al. abstract (see below for publication information)
Ramirez et al. accepted manuscript and figures (Ramirez, B.S. et al., "Active antimetastatic immunotherapy in Lewis lung carcinoma with self EGFR extracellular domain protein in VSSP adjuvant," Int J Cancer, Epub ahead of print 13 July 2006 (DOI: 10.1002/ijc22085))

AMENDMENT TO THE DRAWINGS

Figure 5 has been amended to change the Spanish word "Dia" to "Day". A replacement sheet for Figure 5 reflecting this change is attached.

Attachment: Replacement Sheet for Figure 5.

Active anti-metastatic immunotherapy in Lewis Lung Carcinoma with self EGFR extra cellular domain protein in VSSP adjuvant.

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Key words: EGFR, self-immunization, cancer therapy

Abbreviations: EGFR-ECD (epidermal growth factor receptor extra cellular domain)
VSSP (Very Small Sized Proteoliposomes from ***Neisseria meningitidis)***

Journal Category: Cancer Therapy

A new active specific immunotherapy approach for targeting EGFR, consisting in the immunization with the self EGFR extra cellular domain (ECD) in an appropriated adjuvant (VSSP), circumvented tolerance to this molecule inducing a potent specific immune response with anti-metastatic effect over the EGFR+ Lewis lung carcinoma. These results encouraged us to promote the Her1-ECD/VSSP vaccine for the treatment of patients with EGFR+ tumors.

ABSTRACT:

The Epidermal Growth Factor Receptor (EGFR) plays a central role in regulating neoplastic processes. The EGFR over expression in many human epithelial tumors has been correlated with disease progression and bad prognosis. Passive EGFR-directed immunotherapy but not active specific approaches have already been introduced in medical Oncology practice. Then we wonder if mice immunization with the extra cellular domain of murine EGFR (mEGFR-ECD) in adjuvants can circumvent tolerance to self EGFR, by inducing an immune response with consequent anti-tumor effect. The present study demonstrated that despite mEGFR expression in thymus, strong DTH response was induced by inoculation of mice with the mEGFR-ECD. This self-immunization, using both CFA and Very Small Sized Proteoliposomes from *Neisseria meningitidis* (VSSP), promoted highly specific IgG titers, predominantly IgG2a and IgG2b. Sera from mice immunized with mEGFR-ECD/VSSP not only recognized EGFR+ tumor cell lines by FACS but also inhibited their in vitro growth, even in the absence of complement. Noteworthy, vaccination of mice with mEGFR-ECD/VSSP stimulated a potent anti-metastatic effect in the EGFR+ Lewis lung carcinoma model while reproduction associated side effect were absent. Curiously, mice immunized with the human EGFR ECD (Her1-ECD) in VSSP though induced highly specific IgG antibodies with strong in vitro cytotoxic effect over EGFR+ human cell lines, showed low cross reactivity with the mEGFR-ECD. These results further encouraged the development of the Her1-ECD/VSSP vaccine project for patients with EGFR+ tumors.

INTRODUCTION

The epidermal growth factor receptor (EGFR) belongs to the erbB family of four closely related cell membrane receptors, also known as the Type I Receptor Tyrosine Kinase family: EGFR or HER1/erbB1, first to be molecularly cloned [1], HER2/erbB2, HER3/erbB3 and HER4/erbB4. The four receptors consist of an extra cellular ligand-binding domain (ECD), a transmembrane domain, and an intracellular domain with tyrosine kinase activity for signal transduction. EGFR plays a central role in regulating both development and neoplastic processes. Binding of their specific ligands, such as epidermal growth factor (EGF) or transforming growth factor alpha (TGF-α) among others, induces receptor activation, modulation of cell proliferation and differentiation in normal tissues and tumors. Although expressed in non malignant cells, the EGFR can be found over-expressed or mutated in many human epithelial tumors such as breast [2, 3], lung [4], prostate [5, 6] head and neck [7], colorectal [8], pancreatic [9], bladder [10], vulva and ovarian tumors [11]. This over-expression has been correlated with disease progression and poor prognosis [12, 13]. Activation of the EGFR signaling pathway in cancer cells have shown to enhance cell proliferation, angiogenesis, tumor promotion and metastasis, and to decrease apoptosis. The potential of EGFR-targeted therapies for cancer treatment has increased the development of different passive agents. Passive immunotherapy with specific monoclonal antibodies (MAb) [14, 15] and treatment with tyrosine kinase inhibitor drugs such as Iressa [16, 17] and Tarceva [18], are currently undergoing clinical trials with promising results or are commercially available. On the other hand, active immunotherapy strategies to block the

EGF from binding to its receptor are being clinically tested by vaccinating patients with EGF coupled to P64k recombinant protein from *Neisseria meningitidis* [19].

In addition EGFR-based active specific immunotherapy may be an alternative and complementary approach for the treatment of epithelial tumors, provided that induction of an immune response against self EGFR is feasible. Preclinical studies of both, a DNA vaccine based on xenogenic EGFR-ECD and dendritic cells pulsed with self EGFR-ECD have been recently published, demonstrating the validity of this active immunotherapy [20, 21]. Here an alternative more simple approach, based on vaccination with the mEGFR-ECD protein for exploring the possibility of circumventing tolerance to self EGFR, was proposed. We constructed DNA plasmids encoding the murine EGFR-ECD which were stably transfected in mammalian cells and the corresponding recombinant protein was used for vaccination protocols. Besides, we expressed the Her1-ECD to compare the relative efficacy of self and non-self immunization and for evaluating the immune response specificity to EGFR+ human tumor cells. A strong DTH response and specific IgG titers with a TH1 associated subclass pattern were obtained by inoculation of mice with the self protein in Very Small Sized Proteoliposomes (VSSP) and complete Freund adjuvant. The corresponding immune sera showed *in vitro* anti-tumor effect, inhibiting EGFR+ tumor cells proliferation. mEGFR-ECD vaccination induced a potent anti-metastatic effect in 3LL-D122 Lewis lung carcinoma. Antibodies obtained in mice immunized with Her1-ECD/VSSP evidenced a low cross-reaction with the parent mEGFR-ECD.

MATERIAL AND METHODS

Construction of the expression vector encoding mEGFR-ECD and Her1-ECD

DNA encoding the extracellular domain of murine EGFR was amplified by PCR using total cDNA from mouse liver as template. The sense primer 5′-CGGAATTCCTCTCCCGGTCAGAGATGCGAC-3′ includes EcoRI excision site, the initiation codon and 4 bp from EGFR signal sequence. The antisense primer, 5′-CGGGATCCTCAAGATGGTATCTTTGGCCCAGATG-3′ is complementary to bp 1978- 2000 in 3' region and contains a stop codon (double underlined) and a BamHI excision site (single underlined). The PCR product, a 1.9 kb fragment, was cloned into EcoRI/BamHI sites of the pBluescript KS^+ vector. The fragment encoding for mEGFR-ECD was recovered using HindIII/BamHI enzymes and cloned into the pcDNA3 expression vector (Invitrogene, San Diego, CA, USA), generating the mEGFR-ECD/pcDNA3 plasmid.

DNA encoding the extracellular domain of human EGFR (Her1-ECD) was amplified by PCR using the Her1Δ533/pRK5 plasmid as template. The sense primer 5′-GGGGTACCCTTCGGGGAGCAGCGATGCGA-3′ includes a KpnI excision site (underlined), the initiation codon ATG and 3 bp from the signal sequence of Her1. The antisense primer, 5′-GCTCTAGATCAGGACGGGATCTTAGGCCCA-3′ is complementary to bp 2103-2123 in the 3' region, and contains a stop codon (double underlined) and an XbaI excision site (single underlined). The PCR product, a 1.9-kb fragment, was cloned into KpnI/XbaI sites of the pcDNA 3-expression vector, generating the Her1-ECD/pcDNA3 plasmid.

mEGFR-ECD and Her1-ECD sequences were confirmed, by dideoxy nucleotide sequencing analysis, to be identical with those previously reported [22, 23]. All enzymes were supplied by Boehringer-Mannheim, Penzberg, Germany.

Cell lines

Ehrlich Ascites Tumor (EAT, ECACC No 87032503), 3LLD122, a metastatic variant of Lewis lung carcinoma [24], the murine thymoma EL4 (ATCC TIB-39), human embryonic kidney (HEK293, ATCC CRL-1573), human epidermoid carcinoma A431 (ATCC CRL-1555) and human lung adenocarcinoma H125 [25] cell lines were grown in DMEM (Gibco, USA) supplemented with 10% fetal calf serum (FCS) (Hyclone, Utah, USA), 2mM L-glutamine, 1mM sodium pyruvate, penicillin 100 U/mL / and streptomycin 100 µg/mL (Life Technologies, Grand Island, NY). HEK293 transfectants were adapted to growth in HyQ PF 293 (protein free medium from Hyclone, USA).

Generation of HEK293 transfectants

HEK293 cells were grown in 6 well plates ($1.75x10^5$ cell/mL) and 8 hours later were transfected with 4 µg of mEGFR-ECD/pcDNA3 or Her1-ECD/pcDNA3 plasmids, using the calcium phosphate transfection system. Plates were incubated overnight at 3% CO2, and then at 5% CO2. Transfected cells were selected in medium containing 1,000 µg/mL of G418 (Geneticin, Sigma, USA) starting 48 hours after transfection for the generation of mEGFR-ECD/HEK293 and Her1-ECD/HEK293 stable cell lines. Mock transfection (with pcDNA3 vector) was used as a negative control.

Lectin or antibody mediated precipitations of the recombinant proteins

Supernatant from mEGFR-ECD/HEK293 or Her1-ECD/HEK293 cultures (2 mL) were mixed with 10 µL of lectin-agarose (a lectin from *Triticum Vulgaris*, Sigma, St Louis, USA) or 1 µg of R3 MAb (a MAb specific for human EGFR extracellular domain, CIM, Cuba) plus 20 µl of Protein A-Sepharose (Amershan-Pharmacia Biotech, Uppsala, Sweden), respectively. Samples were gently shaken overnight at 4°C and afterwards centrifuged 1 min at 11 000 g. The precipitated recombinant proteins were separated on SDS-PAGE 7.5%, and visualized by silver staining.

Purification and Immunoblotting of mEGFR-ECD and Her1-ECD

Recombinant proteins were purified from confluent cultures of the respective transfectants by affinity chromatography. EAH-Sepharose 4B (Amershan Pharmacia Biotech, USA) was covalently coupled to human recombinant EGF (hrEGF) (Center of Genetic Engineering and Biotechnology, CIGB, Cuba) or to R3 MAb for mEGFR-ECD and Her1-ECD purification, respectively. Equilibration and washing steps were performed with PBS/NaCl 1M pH 7.0 and protein elution with Glycine 0.2 *M* pH 2.8. Purity was assessed by densitometry, using a personal densitometer SI (Amersham Pharmacia Biotech, USA) and Imag Quant Software. Protein concentrations were assayed by Lowry's method [26].

Purified proteins identity was established by immunoblotting. Recombinant proteins (30 µg) were applied to 7, 5 % SDS-PAGE gels and transferred to PVDF membranes (Gelman, Ann Arbor, MI). After blocking with NEGT buffer (0.15 M NaCl, 5 mM EDTA, 500 mM Tris-HCl (pH 7.5), 0,02 % Tween 20, 0,04 % Gelatin) membranes were incubated with R3 (for Her1-ECD, data not shown) or 7A7 (for mEGFR-ECD) MAbs and proteins were

visualized using horseradish peroxides-conjugated secondary antibodies followed by enhanced chemiluminescence (ECL) (Perkin Elmer Life Sciences, USA).

RT-PCR

Total RNA was isolated from Balb/c or C57BL/6 mice thymus using TRIZOL Reagent (Life Technologies, Grand Island, NY) according to the manufacturer's instructions. The reverse transcription and polymerase chain reaction (RT-PCR) was performed using the SUPERSCRIPT™ One-Step RT-PCR System. Used EGFR and β-actin primers were designed from the published sequences [27]. After PCR amplification, 10 μL of the RT-PCR products were separated by electrophoresis on 1.5% agarose gels and visualized with Ethidium bromide. Total RNAs from murine thymoma EL4 was used as negative control.

Mice and Immunization protocols

Female C57BL/6 mice, aged 8-12 weeks old, were purchased from the National Center for Laboratory Animals Production (CENPALAB, Havana, Cuba). All mice were kept under pathogen-free conditions. Animal experiments were approved by the Center of Molecular Immunology's Institutional Animal Care and Use Committee (CIM, Havana, Cuba).

Mice (n=5 or n=10 for DTH and humoral response studies, respectively) were immunized with 50 μg of either mEGFR-ECD or Her1-ECD in FA adjuvant, complete for the first immunization and incomplete for the rest (Sigma, USA) or in VSSP adjuvant, obtained from the combination of the outer membrane proteins of *Neisseria meningitidis* with GM3 ganglioside, in water/oil (Montanide ISA 51, Seppic, Paris, France) emulsion [28]. Immunizations were made, subcutaneously (sc) for FA adjuvated or intramuscularly (im)

for VSSP adjuvated preparations, on days 0, 14 (for the DTH study) and 0, 14, 28, and 42 (for humoral response studies). Sera were extracted on days 0, 21, 35, 56. Control groups received PBS/FA or PBS/VSSP.

DTH test

Seven days after the last immunization, mice were sensitized by intradermal injection with 50 μg of mEGFR-ECD in 50 μL of PBS in the right hind foot pad and the same volume of PBS in the left foot pad. After 48 hours mice foot swellings were measured using a plethysmometer (Ugo Basile, VA, Italy). Mice immunized with 100 μg of Keyhole Limphet Hemocyanin (KLH), (Sigma, Aldrich, USA) in FA and sensitized with KLH in PBS were used as positive controls while injected with PBS/FA and sensitized with mEGFR-ECD were considered as negative controls. Differences in DTH between treatment groups were statistically validated by Kruskal Wallis and Dunn's Multiple Comparison test.

Enzyme immunoassay

Microtiter plates (High binding, Costar, USA) were coated with 10 μg/mL of mEGFR-ECD or Her1-ECD in carbonate buffer, 0.1 *M*, pH 9.6, and incubated overnight at 4°C. Plates were blocked with 5% calf serum in PBS/Tween-20, and sera dilutions in duplicate, from immunized mice (n=10), or pre-immune sera (as negative control) were incubated 1 hour at 37°C. Alkaline phosphatase conjugated goat anti-mouse IgG antibody (Sigma, USA) was added and incubated 1 hour at 37°C. After addition of P-nitrophenylphosphate (1 mg/mL) (Sigma, USA) the Optical Density (OD) was measured at 405 nm using a micro well system reader (Organon Teknica Inc., Salzburg, Austria). All washes were made with PBS/Tween-20. The Mann Whitney U test was used to assess statistical differences

between individual time points in the humoral response kinetic. ELISA test background was two times the OD at 405 nm of pre-immune sera, which coincide with the OD value for PBS.

For determination of serum IgG subclasses secondary isotype-specific biotinylated rat anti-mouse IgG1, IgG2a, IgG2b, or IgG3 antibodies were used (PharMingen, USA). Optimal secondary reagents dilutions were established by ELISA with 14F7 (IgG1 specific for NGcGM3), T3 (IgG2a specific for CD3) and T4 (IgG2b specific for CD4) MAbs (CIM, Cuba) while R24 MAb (IgG3 specific for GD3) (kindly provided by Dr. Philip O. Livingston, Memorial Sloan Kettering Cancer Center, NY, USA). Unpaired *t* test was used to check statistically significant differences between sera dilutions.

ELISPOT

Specific inguinal lymph (LN) nodes spot forming cells (SFC) were obtained from mice immunized with Her1-ECD/VSSP and tested by Enzyme Linked Immunospot (ELISPOT) as previously described [29], with some modifications. Maxisorp 96 well plates (Nunc, USA) were coated with 10 μg/ml of Her1-ECD or mEGFR-ECD, in 50 μl carbonate buffer (pH 9.8) at 4°C, overnight. After blocking with 5% BSA in PBS, different dilutions of pooled LN cells were incubated in triplicate 6 hr at 37°C in a 5% CO_2 incubator. Antibodies secreted by individual cells were revealed as spots by the stepwise addition of 1, 5 μg/ml of alkaline phosphatase-conjugated goat anti-mouse IgG (Fcγ) or IgM (Fcμ) antibodies (Jackson Immunoresearch laboratories Inc, West Grove, PA) and the addition of 1mg/ml of 5-bromo-4-chloro-3-indolyl phosphate (BCIP) substrate (Sigma, San Louis, MO) in 0.1 M AMP buffer, pH 10.5, containing 0.6% agarose. Plates were incubated overnight at 4°C and

the results were scored the next day by counting the number of specific spot forming cells in a stereoscopic microscope. LN cells from mice injected with PBS/FA were used as negative control.

FACS Analysis for EGFR recognition

Cells were stained with sera from immunized mice (1/200 dilution) followed by FITC-goat anti-mouse IgG (Jackson, Immunoresearch laboratories Inc, West Grove, PA). Up to 10,000 cells were acquired using a FACScan flow cytometer and analyzed using the CellQuest software (Beckton Dickinson, San Jose, CA, USA). PCR and 7A7 MAb [30], which is specific for mEGFR-ECD, were used to confirm EGFR expression in murine cells. Human EGFR expression in the corresponding cells was confirmed with R3 MAb. EL4 murine cell line and human lymphocytes were used as negative control cells.

Growth Assay

Flat bottomed 96-well microculture plates were seeded with 10^4 cells in 100 µL/well and grown in DMEM supplemented with 1% FCS in the presence of sera dilutions. After 48 h of incubation at 5% of CO2, cells' viability was measured by the modified colorimetric MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazodium bromide) assay [31, 32]. Media were replaced by 100 µL/well of MTT (1 mg/mL) and plates were incubated under culture conditions for 4 hours. Formazan crystals were dissolved by addition of 100 µL/well of dimethyl sulphoxide followed by 30 minutes incubation at 37°C. Absorbance (OD) was measured at 540 nm using a microplate spectrophotometer and the reference wavelength (620 nm) OD subtracted. Background control contained only culture medium without cells. Cells without treatment were included as a maximum cell growth point. R3 and 7A7 MAbs

were used as positive controls for human and murine cells lines, respectively. Statistical differences in the *in vitro* viability assay were evaluated by the Unpaired *t* test.

Cytotoxicity Assay

Flat bottomed 12-well microculture plates were seeded with $5x10^5$ cells in 100 µL/well and grown in DMEM supplemented with 1% FCS in the presence of diluted sera (1/10). Sera from non-immunized mice were used as control for unspecific complement mediated cytotoxicity. To measure specific complement independent cytotoxicity, immune sera were heated 30 minutes at 56°C and after 24 hours of incubation at 5% of CO2, death cells were counted by FACS using Propydium Iodide. Cells without treatment were included as control for minimum death cell. R3 and 7A7 MAbs were used as positive controls for human and murine cells lines, respectively.

Tumor challenge assay

C57/BL6 mice (n=10) were immunized, intramuscularly, 3 times biweekly with 100 µg of mEGFR-ECD/VSSP or PBS/VSSP. One day before the second immunization, mice were challenged with $2x10^5$ tumor cells, subcutaneously, in the foot pad. Three weeks later, tumors reached 0.8 cm and were surgically removed. 21 days after surgery, mice were sacrificed and spontaneous lung metastases quantified by weighing the lungs. Statistical differences between groups were determined by Unpaired *t* test.

Reproductive side-effects studies

Female Balb/c mice (n=10) were immunized with mEGFR-ECD/VSSP or PBS/VSSP (control group) as previously described. After checking the induction of specific antibodies against mEGFR-ECD, mice were mated with non-immunized male animals. Fertility

(number of mice completing pregnancy), number of pups, pups' birth weights, and certain postnatal developmental features such as eyes opening, hair growth and incisor eruption were observed. The Mann Whitney U test was used to test statistically significant variations in the reproduction parameters between treated and control animals.

Statistical Analyses

Variance homogeneity and data normal distribution were analyzed by Bartlett's and Bonferroni tests, respectively, using the SPSS version 10.0 Software. All statistical tests (Kruskal Wallis and Unpaired *t* tests) were two-sided and conducted using the Graph Pad Prism version 4.00 Software. A probability value of $p<0.05$ was considered as statistically significant.

RESULTS

mEGFR-ECD and Her1-ECD expression and purification

cDNAs encoding mEGFR-ECD and Her1-ECD were successfully cloned into the pcDNA3 expression vector (Fig. 1A) and transfected in HEK293 cells as previous description. Expression of the soluble recombinant proteins by stable HEK293 transfectants was checked by lectin-agarose precipitation (mEGFR-ECD) or immune-precipitation with R3 MAb (Her1-ECD). In each case, as expected, a 105 kDa protein band was displayed but not for mock transfection, as determined by SDS-PAGE. Figure 1B showed the corresponding results for the mEGFR-ECD. mEGFR-ECD identity was further confirmed by affinity chromatography (with hrEGF-EAH Sepharose) purification combined with SDS-PAGE and western blotting, showing again the 105 kDa protein band (Fig. 1C). Achieved protein

purity was 98% after only one purification step as determined by densitometry (data not shown).

mEGFR is expressed in thymus

As has been previously reported from studies with rat cell lines [33] and human thymus [34], the EGFR presence in mice thymus was demonstrated by RT-PCR. The corresponding EGFR cDNA band (Fig. 2) became apparent when thymuses from C57BL/6 and Balb/c mice were analyzed, but not from murine thymoma EL4, used as a negative control.

mEGFR-ECD immunization induces DTH response

Generation of specific DTH responses was considered as a primary endpoint for the mEGFR-ECD vaccine (emulsified in FA) ability in immunizing mice. Mice were subcutaneously injected with mEGFR-ECD/FA and then sensitized with 50 μg of the nominal antigen. After 48 hours mice foot swellings were measured and inflammation scores in the vaccinated group animals were higher than those of the negative control group ($p<0.05$, Dunn's multiple comparison test). Noteworthy, DTH responses induced in mice vaccinated with either mEGFR-ECD or KLH, a strongly immunogenic protein, were similar ($p>0.05$) (Fig. 3).

mEGFR-ECD immunization induces a strong and long lasting specific humoral response

Vaccine related humoral responses were explored by immunizing C57BL/6 mice four times, biweekly, with 50 μg of mEGFR-ECD in two different adjuvants: FA, the reference adjuvant, and VSSP, a new product already clinically tested in humans. Inoculated mice developed high serum IgG antibody levels against the immunizing protein, which increased

with successive immunizations, for both adjuvant formulations (Fig. 4). Indeed the mEGFR-ECD /VSSP vaccine induced higher antibody titers than the FA one in each sera collection day (Mann Whitney U test, $p<0.05$). 8 of 10 mice (80%), immunized with mEGFR-ECD/VSSP, showed specific antibody titers above 1/40 000 by day 56, even reaching values up to 1/160 000, whilst only 2 of 10 mice (20%) rose titers above 1/40 000 in the mEGFR-ECD/FA immunized group. One year after having finished the immunization schedule and without antigen recall the sera specific IgG levels decreased in both groups of mice, although an appreciable response was still detected in 100% of animals (1/1000 and 1/100 sera dilutions) (data not shown).

Administration of mEGFR-ECD in FA or VSSP to Balb/c mice produced elevated IgG titers in 100% of animals, similarly to what was observed in the C57Bl/6 case (data not shown).

Vaccination with mEGFR-ECD polarizes systemic immunity to a TH1 pattern

Consistent with previous results demonstrating that complete FA preferentially promotes a TH1 type response to the accompanying antigen [35], elevated levels of IgG2a, IgG2b, and IgG1, were detected in day 21 sera corresponding to mice immunized with mEGFR-ECD/FA. As shown in Figure 5, while no differences in IgG2a levels were found in sera of mice vaccinated with FA or VSSP formulations (Unpaired *t* test, $p>0.05$), the use of VSSP promoted the induction of higher levels of IgG2b ($p<0.05$).

Her1-ECD/VSSP immunization generates specific B cell clones with low cross-reactivity to the mEGFR-ECD

We wonder if immunization with the xenogenic EGFR-ECD could induce antibodies reacting with the self EGFR. C57BL/6 mice were immunized with 50 μg of Her1-ECD in FA or VSSP. All immunized mice, independent of the used adjuvant, developed high IgG antibody titers against the human protein (1/320 000) by day 56 (data not shown). Alternatively, ELISA experiments reflected an evident but low cross-reactivity with the murine protein by day 21 (Fig. 6A). This low cross-reactivity against the mEGFR-ECD was confirmed by ELISPOT assay. 62 IgG and 6 IgM secreting specific spot forming cells (SFC) in 10^6 LN cells were found in LN of Her1-ECD immunized mice while only 21 IgG secreting SFC/10^6 LN cells cross-reacted with the mEGFR-ECD (Fig. 6B).

Immune sera recognizes full length EGFR by FACS

To check whether immunizations with a truncated EGFR affected the recognition of the full length EGFR in its native conformation on the cell surface, EGFR+ cells were analyzed by FACS. EAT [36] and 3LL-D122 murine cell lines were positively stained by sera from mice immunized with mEGFR-ECD/VSSP (Fig. 7A). Besides, sera from mice immunized with Her1-ECD/VSSP readily reacted with A431 and H125 cell lines (Fig. 7B). Sera from control mice, immunized with PBS/VSSP, neither recognized murine nor human EGFR+ cells.

Immune sera inhibit EGFR+ tumor cells growth and possess cytotoxic effect

In order to determine whether immunization with mEGFR-ECD/VSSP or Her1-ECD/VSSP can generate serum antibodies affecting murine or human tumor cells growth *in vitro,* the MTT-viability assay was performed. Incubation of 3LL-D122 or H125 cells with sera obtained from mice immunized with mEGFR-ECD/VSSP or Her1-ECD/VSSP,

respectively, decreased the number of viable cells if compared with pre-immune sera after 48 hours and this effect was sera dilution dependent (Unpaired t test, $p<0.05$) (Fig. 8A). In addition, the immune sera *in vitro* cytotoxicity over EGFR+ cells was evaluated after treating cells for 24 hours with the corresponding complement inactivated samples, following Propydium Iodide staining. FACS analysis showed that treatment of 3LL-D122 cells with inactivated sera produced 55, 83% of death cells, suggesting that a complement independent cytotoxicity mechanism is operating (Fig. 9A). In parallel, H125 cells were treated with sera from mice immunized with Her1-ECD/VSSP, and the effects over the cells growth evaluated. Incubation with immune sera decreased cells' viability, compared with pre-immune sera (Fig. 8B), and manifested cytotoxic effects (FACS) (Fig. 9B).

Anti-metastatic effect of mEGFR-ECD/VSSP vaccination

To investigate whether the autologous vaccination can protect individuals from metastatic widespread, mice were immunized with the mEGFR-ECD in VSSP, and one day before the second immunization, challenged with $2x10^5$ tumor cells in the foot pad. Three weeks after malignant cells inoculation, tumors were surgically removed. 21 days after surgery, mice were sacrificed and the spontaneous lung metastases quantified by weighing the lungs. As shown in Figure 10, vaccination of mice significantly reduced lung metastasis ($p<0.01$), compared with animals in the control group.

Absence of reproductive side effects in mice immunized with mEGFR-ECD/VSSP

The potential side effects of "self" immunization in humans were stressed by examining the appearance of possible toxic symptoms in female mice immunized with the mEGFR-ECD in VSSP. After the vaccine inoculation animals were mated and their progeny studied.

Pregnancies rates were 5 out of 10 in the immunized group, while 3 out of 10 in control mice. The median number of pups per litter in both groups was 6 (range 5 to 7). Features like newborns' weights, hair growth, eyes opening and incisor appearance didn't comparatively differ (Mann Whitney U test, $p>0.05$) (Table 2). On the other hand, a group of mice were observed for one year after fulfilling the immunization protocol, and the vitality, temperature, and food intake were completely normal, without changes in functional hepatic parameters if compared with non-immunized mice (data not shown).

DISCUSSION

Despite the EGFR wide expression in the organism, it can be considered as a tumor-associated antigen (TAA) due to its over-expression in many epithelial tumors[37], its implication in tumor growth, and correlation with bad prognosis[12]. For that reason the EGFR has become an attractive target for cancer therapy and many attempts are currently ongoing, using this molecule as target for passive therapy with tyrosine kinase inhibitors and monoclonal antibodies [38-40]. Our results demonstrated that the murine EGFR extracellular domain, in an appropriate adjuvant, is enough immunogenic in mice to promote a strong anti-metastatic effect in a relevant EGFR+ tumor model, highlighting this particular approach as an attractive new strategy for cancer treatment.

TAA tolerance represents a significant challenge for effective immunotherapy of human cancer and in any successful vaccine strategy this issue should be conveniently addressed in advance. The experimental demonstration that breaking "self" tolerance is possible has been recently published and is generally accepted under the principle that "self"

recognition is a physiological phenomenon [41]. The fact that the EGFR is widely distributed in normal epithelial tissues hardly suggests that this molecule could be a very poor immunogen, introducing additional difficulties to any vaccine design. In our vaccine approach the construction of chimerical molecules was avoided, introducing instead potent TH1 adjuvants. Emulsifying the recombinant protein either in FA or VSSP (a product already clinically tested in humans) renders a rather simple formulation. As the primary endpoint for the vaccine induced mEGFR-ECD immunogenicity, the induction of DTH was considered. Surprisingly, mEGFR-ECD/FA vaccination promoted severe inflammations in mice foot pads after the specific sensitization, similarly to those mice previously immunized with KLH/FA and sensitized with this "foreign" protein.

Immunization of mice with the mEGFR-ECD in FA or VSSP also stimulated the specific humoral immunity, characterized by elevated IgG antibody titers, successively incremented with re-immunizations, an indicative of a mature response. Both adjuvants influenced IgG subclasses distribution in favor of IgG2a and IgG2b, an indirect indication of TH1 differentiation. Particularly higher levels of IgG2b were associated with VSSP formulations. An indicative that the immune reaction against this kind of "self" proteins is limited came from other member of the EGFR family, Her2. Monkeys were successful immunized only after 6 immunizations with the Her2-ECD, formulated in the powerful adjuvant Detox, and specific IgG titers never reached 1/10 000 [42], a value lower than that induced in mice with the mEGFR-ECD in FA or VSSP. Besides, Disis et al [43] have shown that a Her2 neu-peptide-based vaccine, but not a whole-protein vaccine, can elicit humoral and cellular responses in rats. The EGFR is a tolerated "self"-antigen for which a specific T cells thymic deletion mechanism could be operating, requiring the presence of the auto-

antigen in the thymus [44, 45]. The EGFR thymic expression, while early reported in humans [34] and rats [33] was confirmed in our lab for Balb/c and C57Bl/6 mice by RT-PCR. Similarly to what has been reported in normal people, a total absence of anti-EGFR natural auto-antibodies was also observed in mice sera by ELISA and FACS. Nevertheless, evidences coming from cancer patients indicated that the presence of discrete serum anti-EGFR natural antibodies can be detected with certain frequency [46]. The same observations have been reported for breast cancer patients in which anti-Her2 antibodies and CTL could be measured [47], lacking enough efficacy in preventing tumor progression. This "natural" immunity to Her2, present only in a minority of patients over-expressing the receptor, is of low magnitude [48]. The low natural immune responses to Her1 and Her2 in cancer patients means that any associated target-directed therapeutic vaccine must efficaciously stimulate naïve B and T lymphocytes. In fact, Her2 vaccines, constructed with synthetic peptides mixed with granulocyte-macrophage colony stimulating factor (GM-CSF) as adjuvant, have been clinically tested in breast, ovarian and lung cancer patients over-expressing Her2 and 68% of them developed T cell immunity against the self Her2 protein [49].

More likely explanations for the unusually strong immunogenicity of the autologous Her1 protein observed in this study might be the full length EGFR truncation, the adjuvant conditioning of the antigen presentation context or both. EGFR truncation could modify the T cells repertoire immunodominance, favoring the presentation of cryptic determinants [50]. In this case protein truncation didn't affect the full length EGFR recognition in its natural conformation in the cells by the vaccine induced serum antibodies, as determined in FACS experiments. This result suggest certain differences with the Her2 model, in which specific antibodies were undetectable in sera obtained from rats immunized with the rat Her2

intracellular domain (ICD), while CTL and antibodies with degenerated specificities for the human and rat Her2/neu were produced when the inoculated immunogen was the highly homologous foreign human ICD [51].

The use of potent TH1-type adjuvants in combination with poorly immunogenic "self" proteins to promote a pro-inflammatory context for loosing the regulatory cells circuit was an attractive tested idea. In this sense and as usual in experimental vaccine approaches complete FA was selected as reference TH1 adjuvant [35]. While complete FA can't be used in human vaccines, a new already clinically tested adjuvant (VSSP) with peculiar immune-modulatory properties was introduced in the EGFR-ECD vaccines. VSSP monotherapy in mice induced elevated IgG levels with a TH1 related pattern against GM3, a poorly immunogenic ganglioside, [52] and dendritic cell maturation with IL-12 production [52] which in turn is pivotal for pro-inflammatory responses [53, 54].

Although the amino acid sequence homology between human and murine EGFR-ECDs is about 87%, sera obtained from mice immunized with the Her1-ECD formulation were unable to appropriately react with the murine protein, while only 1/3 of the vaccine stimulated B cells, secreting specific IgG antibodies, cross-reacted with the mEGFR-ECD. The finding that serum specific antibodies, induced in mice by immunization with the corresponding EGFR-ECDs in VSSP, caused the slow-growing of EGFR+ tumor cells and also promoted a strong target-directed complement independent cytotoxic effect, emphasize the quality of the vaccine induced immune response.

Further results associated with the *in vivo* mEGFR-ECD/VSSP anti-metastatic effect in the EGFR+ 3LL-D122 Lewis lung carcinoma model are encouraging if the intermediate

character of the selected experimental setting (rather therapeutic than prophylactic) was considered. Even though in this case tumor cells were inoculated into mice foot pads just after vaccine priming, supplying afterwards the two booster injections, a significant decrease in lung metastases number, after primary tumor surgical removal, was evident.

One week after the third vaccine administration and coincident with the surgical removal of primary tumors, specific IgG titers in mice sera were elevated as noticed from the humoral response kinetics, probably suggesting that the induced antibodies might have a role in avoiding tumor cells' dissemination or in turn the growth of malignant cells already lodged in the lungs, or both. Future studies stressing the relative contribution of humoral and cellular immunity in the vaccine induced anti-metastatic effect, through hyper immune serum transfer and the appropriated lymphocyte subsets depletion experiments are currently ongoing. The "spontaneous metastasis" 3LL Lewis Lung carcinoma model, employed in this study, is rather significant because of the most likely resemblance to the real clinical situation in which surgeons frequently remove patients' primary tumors successfully but, unfortunately and more commonly, disease spreading will follow. Indeed, avoiding distant metastatic dissemination could be the most appropriate task for effective cancer vaccines. Interestingly, other two active immunotherapy approaches targeting the EGFR have shown recently *in vivo* efficacy in the Lewis lung carcinoma model but in a primary tumor scenario. A DNA vaccine, [20] based on the xenogenic EGFR-ECD gene and a "self" EGFR ECD protein pulsed dendritic cells vaccine [21] were able to keep alive 60 % of mice preventatively injected and afterwards challenged with LL/2c tumor. Although together all these results strongly suggests that EGFR could be a significant target, not only for passive but also for active immunotherapy, another crucial remaining question is the better vaccine

approach to follow up in the upcoming future clinical trials. A relevant learning from the present work is that just the use of the recombinant "self" protein in a potent adjuvant, like VSSP, could be appropriate for establishing an anti-tumor immunity in patients with EGFR+ tumors, indicating the existence of a new opportunity in this particular target different from sophisticated vaccine technologies like the dendritic cells approach, or up to now ineffective vaccine formulations in humans like naked DNA.

EGFR-targeted therapies are expected to produce side effects related to the induction of autoimmunity. In fact, some side effects as skin rash have been reported for some related drugs [55], but important toxic effects have not been found for the majority of the different approaches tested in clinical trials. As an example THERACIM, an anti-EGFR monoclonal antibody (humanized R3, CIM, Cuba) has been clinically tested (Phase II trials), in combination with radiotherapy, in head and neck cancer patients, raising up to 600 mg/cycle, without the detection of skin rash symptoms. Although it has been reported that EGFR gene expression inhibition is critical for cancerous cell growth but not for normal cells, [56] active immunization, providing a long lasting specific immune response, should be carefully monitored for possible side effects. The conducted immunization experiments with mEGFR-ECD/VSSP showed that while one year after the last vaccine booster anti-mEGFR-ECD IgG low levels were detectable in most animals, signs of toxicity were absent and functional hepatic parameters behave as in naïve mice.

Induction of EGF deficiency in rats affects the development of fetal but not adult tissues [57]. Considering the role of EGF in the epigenetic regulation of fetal and neonatal development, we studied the effect of anti-EGFR immunity in female mice fertility and their progeny. Marked side effects in these animals as a consequence of vaccination were not detected.

We conclude that "self" EGFR-ECD is rather immunogenic in FA or VSSP contexts. Taken together, the immunization approach described here may be an attractive and novel strategy for EGFR+ cancer active immunotherapy. .

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Figure captions

Figure 1: **Construction and functionality of the expression vector and purification of the mEGFR-ECD: A)** DNAs encoding the mEGFR-ECD or the Her1-ECD were inserted into the pcDNA3 expression vector. **B)** mEGFR-ECD/pcDNA3 construct was verified by sequencing and protein expression was checked by precipitation with lectin-agarose from supernatants of the HEK293 transfectant, and visualized in 7.5% SDS-PAGE gels with silver staining. Mock transfection (pcDNA3) was used as control. **C)** mEGFR-ECD purification was afforded by affinity chromatography with hrEGF-EAH Sepharose (left) and protein identity confirmation by western blotting (right) using 7A7 MAb. An isotype control MAb was used as negative control.

Figure 2: EGFR is expressed in mice thymus tissue. Thymuses from C57/Bl6 and Balb/c mice were analyzed by RT-PCR for EGFR expression. Total RNA was extracted from thymuses of C57Bl/6 or Balb/c mice and from EL4 tumor (negative control), using TRIZOL reagent. 1 μg of RNA was reversed-transcribed to cDNA and the specific fragments amplified by PCR. β-actin mRNA served as an internal control.

Figure 3: mEGFR-ECD specific DTH response. Vaccine induced DTH response was assayed by immunizing C57Bl/6 mice two times with 50 μg of mEGFR-ECD/FA. 50 μg of KLH/FA was used as positive control and PBS/FA as negative control. Mice included in the first and third groups were sensitized 7 days later with the mEGFR-ECD in PBS while animals belonging to the second group with KLH. mEGFR-ECD/FA treated mice showed higher foot pads inflammations (Dunn's Multiple Comparison test, $p<0.05$) than PBS

control mice, but similar (Dunn's Multiple Comparison test, $p>0.05$) than those in KLH group. A representative experiment from two independent ones is shown.

Figure 4: Kinetics of the anti-mEGFR-ECD humoral response. Mice were immunized four times with 50 μg of the mEGFR-ECD in VSSP or FA, biweekly. Antibody titers were quantified by ELISA in sera collected on days 0, 21, 35 and 56. Data was log transformed (1+1/titer) for graphic representation. While absent before the first vaccine administration (day 0), an increased antibody presence was detected after immunizations, preferentially when VSSP was used as adjuvant (Mann Whitney U test, $p<0.05$). Immunization days are represented by arrows. A representative experiment from three independent ones is shown.

Figure 5: IgG subclasses induced by immunization. Serum IgG subclasses pattern in C57Bl/6 mice after two inoculation with 50 μg of the mEGFR-ECD in VSSP or CFA, biweekly, was measured by ELISA with samples collected on day 21, and diluted 1/10 000. Each point represents the mean absorbance value of duplicate samples in individual mice (n=5). IgG2b levels were higher (Unpaired *t* test, $p<0.05$) in the group immunized with mEGFR-ECD/VSSP while not differences where found for IgG2a. A representative experiment from three independent ones is shown.

Figure 6: Immunization with Her1-ECD/VSSP mobilizes B cell responses: Mice were immunized two times with 50 μg of Her1-ECD/VSSP, biweekly. **(A)** Specific IgG antibodies against the Her1-ECD (II) and their cross-reaction with mEGFR-ECD (I) were assayed by ELISA employing sera collected on day 21. **B)** Specific spot forming cells, secreting IgG and IgM antibodies against the Her1-ECD and their cross-reaction with the

mEGFR-ECD were measured by ELISPOT. A representative experiment from two independent ones is shown.

Figure 7: Immune sera recognize EGFR+ tumor cells. A) 1/200 sera dilutions, obtained from mice immunized with mEGFR-ECD/VSSP, reacted with 3LL-122 and EAT cell lines (black line) but not with EL4 cells. **B)** 1/200 sera dilutions, obtained from mice immunized with Her1-ECD/VSSP, reacted with A431 and H125 cell lines (black line) but not with human lymphocytes. Sera from mice immunized with PBS/VSSP (gray line) were used as negative control.

Figure 8: Immune sera inhibit EGFR+ tumor cells growth. A) 3LL-D122 cells or **B)** H125 cells were grown in the presence of sera obtained from mice immunized with the mEGFR-ECD or the Her1-ECD, respectively. Pre-immune sera were used as negative controls. After 48 hr, cells' viability was measured by the MTT colorimetric assay. Both kinds of hyper-immune sera were able to significantly decrease viable cells number (Unpaired t test, $p<0.05$). Each bar represents the mean absorbance ± SD of two independent experiments.

Figure 9: Immune sera cytotoxic effect over EGFR+ cells: A) 3LL-D122 and **B)** H125 cells were incubated 24 hours with inactivated-complement sera from mice immunized with mEGFR-ECD/VSSP or Her1-ECD/VSSP, respectively. The immune sera cytotoxic effect was determined by FACS analysis. 7A7 (50% of cytotoxicity) and R3 (25% of cytotoxicity) MAbs were used as positive controls for 3LL-D122 and H125 cells, respectively. This experiment is representative of two independent ones.

Figure 10: Anti-metastatic effect of mEGFR-ECD/VSSP vaccination: Mice were immunized three times with 100 μg of mEGFR-ECD/VSSP, biweekly. One day before the second immunization, mice were challenged with $2x10^5$ 3LL-D122 tumor cells in the foot pad. Three weeks after malignant cells inoculation, tumors were surgically removed and 21 days later mice were sacrificed and the spontaneous lung metastases quantified by weighing the lungs. In vaccinated animals lung weights were significantly reduced ($p<0.01$, Unpaired *t* test) compared with the corresponding control group. This experiment is representative of two independent ones.

Table 1

Response frequency and IgG titers* in individual animals by day 56 after immunization with the mEGFR-ECD, using FA or VSSP as adjuvants

Treatment groups	Response Frequency	1/ IgG titer						
		2 500	5 000	10 000	20 000	40 000	80 000	160 000
mEGFR-ECD in FA	10 / 10	1	1	3	3	1	1	
mEGFR-ECD in VSSP	10 / 10		1		1	2	4	2

* Assayed by ELISA

Table 2

Pregnancy and newborn reproductive parameters measured after mEGFR-ECD/VSSP immunization

Group	Fertility	Number of pups per litter	Weigh of one day old pups (g) (Mean ± SD)	Hair growth (d.a.b.)*	Eyes opening (d.a.b.)	Incisor eruption (d.a.b.)
Treated	5/10	5-7	1.298±0.03	5-7	13-15	10-13
Control	3/10	5-7	1.33±0.07	5-7	13-16	10-13

* d.a.b. = Days after birth

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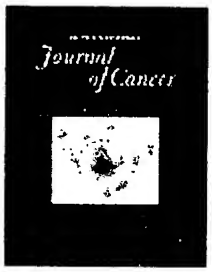

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Cancer Therapy

Active antimetastatic immunotherapy in Lewis lung carcinoma with self EGFR extracellular domain protein in VSSP adjuvant

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KEYWORDS

EGFR • self-immunization • cancer therapy

ABSTRACT

The epidermal growth factor receptor (EGFR) plays a central role in regulating neoplastic processes. The EGFR overexpression in many human epithelial tumors has been correlated with disease progression and bad prognosis. Passive EGFR-directed immunotherapy, but not active specific approaches, has already been introduced in medical oncology practice. Then we wonder if mice immunization with the extracellular domain of murine EGFR (mEGFR-ECD) in adjuvants can circumvent tolerance to self EGFR, by inducing an immune response with consequent antitumor effect. The present study demonstrated that despite mEGFR expression in thymus, strong DTH response was induced by inoculation of mice with the mEGFR-ECD. This self-immunization, using both CFA and very small sized proteoliposomes from *Neisseria meningitidis* (VSSP), promoted highly specific IgG titers, predominantly IgG2a and IgG2b. Sera from mice immunized with mEGFR-ECD/VSSP not only recognized EGFR+ tumor cell lines by FACS, but also inhibited their *in vitro* growth, even in the absence of complement. Noteworthy, vaccination of mice with mEGFR-ECD/VSSP stimulated a potent antimetastatic effect in the EGFR+ Lewis lung carcinoma model, while reproduction-associated side effects were absent. Curiously, mice immunized with the human EGFR-ECD (Her1-ECD) in VSSP though induced highly specific IgG antibodies with strong *in vitro* cytotoxic effect over EGFR+ human cell lines, showed low cross-reactivity with the mEGFR-ECD. These results further encouraged the development of the Her1-ECD/VSSP vaccine project for patients with EGFR+ tumors.